Endocr (2010) 37:55–61
DOI 10.1007/s12020-009-9267-y

ORIGINAL ARTICLE

Raloxifene inhibits bone loss and improves bone strength through an Opg-independent mechanism

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Received: 23 April 2009 / Accepted: 11 September 2009 / Published online: 6 November 2009

Abstract The osteoblast-derived paracrine factor osteoprotegerin (OPG) is considered to play a key role in inhibition of osteoclast formation and activity. Recently, raloxifene, a nonsteroidal benzothiophene, was found to exert anti-resorptive effects via modulating OPG expression in osteoblasts. To explore whether raloxifene regulates bone metabolism via an OPG-dependant pathway in vivo, we investigated the effects of raloxifene on bone loss in *Opg*-deficient mice. The results show that bone mineral density and bone strength are increased in mice deficient for Opg after treatment with raloxifene for 30 days. Histomorphometric analysis shows that raloxifene can increase bone trabecular area and decrease the number of osteoclasts in $Opg^{-/-}$ mice. Moreover, raloxifene reduces *Rankl* transcription and serum level of Rankl, which is dramatically increased in *Opg* knockout mice. These results suggest that raloxifene-induced inhibition of bone resorption may be independent of Opg pathway in mice.

Keywords Raloxifene · Osteoprotegerin · Bone resorption · Osteoclast · *Opg* knockout mice

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Introduction

Decrease of estrogen in postmenopausal women results in an increased bone turnover with bone resorption exceeding bone formation, leading to a general loss of bone mass [1]. Estrogen replacement therapy (ERT) can contribute to maintenance of skeletal mass and reduce the risk of fractures in postmenopausal women [2, 3]. However, the side effects induced by administration of estrogen limit its clinical application. New synthetic nonsteroidal compounds, the selective estrogen receptor modulators (SERMs), have been recently developed and shown to possess estrogen receptor agonistic or antagonistic selectivity in specific target tissues [4–7]. Currently, only one SERM, raloxifene is approved for the prevention and treatment of osteoporosis. It is a benzothiophene SERM that specifically activates biological responses in bone tissue without stimulation of mammary gland and uterus [8, 9].

Although raloxifene was used as an anti-resorption agent, its mechanism of inhibitory effect on bone resorption is not fully elucidated. Osteoclasts are the only somatic cells with bone-resorbing capacity and have a critical role in the pathogenesis of osteoporosis. It has been shown that, similar to estrogen, raloxifene inhibits osteoclast formation

in human and mouse bone marrow cultures in vitro [10, 11]. Receptor activator of nuclear factor (NF)-κB ligand (RANKL) is a membrane-bound protein of the tumor necrosis factor ligand family that is expressed on the osteoblast cell surface and has been shown to play a major role in osteoclast differentiation along with macrophage colony stimulating factor. As a decoy receptor for RANKL, osteoprotegerin (OPG) can prevent its interaction with the cognate receptor RANK. Therefore, OPG is a key factor for maintaining the balance between bone resorption and bone formation. Raloxifene can stimulate the release of OPG in osteoblasts in vitro [12, 13]. Moreover, clinical data indicate that raloxifene can improve osteoporosis through an increased OPG production by osteoblasts [14].

To further explore whether the anti-resorption effect of raloxifene is dependent of the *Opg* pathway in vivo, *Opg* knockout mouse model was used to illustrate the relationship between drug-regulated bone metabolism and the *Opg* pathway. The results show that bone mass and bone strength are increased in mice deficient for Opg after treatment with raloxifene, suggesting that raloxifene inhibits bone resorption is via an *Opg*-independent pathway in vivo.

Results

Genotyping of *Opg* knockout mice

Opg knockout mice were generated through homologous recombination and supplied by Shanghai Research Center for Model Organisms. Genomic DNA from mouse tails was used for genotyping by PCR [15]. When using a set of three paired primers in all PCR reactions, wild-type (580 bp) and $Opg^{-/-}$ mice (620 bp) showed only one band with expected size, and heterozygous mice ($Opg^{+/-}$) mice exhibited both (Fig. 1).

Raloxifene increases bone mineral density in $Opg^{-/-}$ mice

In control group (treated with placebo, PEG 300), bone mineral density (BMD) in both lumbar vertebrae and femurs was significantly decreased in $Opg^{-/-}$ mice when

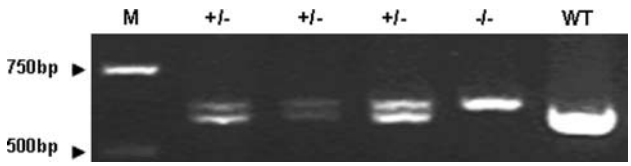

Fig. 1 PCR genotyping for *Opg* knockout mice. Genomic DNA from mouse tails was used to amplify the fragments derived from the wild-type (500 bp) and mutant (750 bp) alleles by using three specific primers

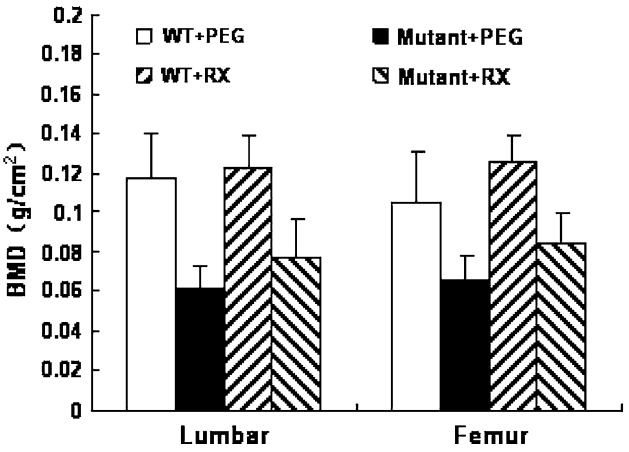

Fig. 2 Raloxifene increases BMD in $Opg^{-/-}$ mice. Age- and sex-matched WT and $Opg^{-/-}$ mice were treated with either PEG 300 (*PEG*) or raloxifene (*RX*) for 1 month. The BMD of both lumbar vertebrae and femurs was measured by DXA method. The results are expressed as mean ± SD ($n = 10$)

compared to that of age- and sex-matched WT mice (lumbar vertebrae, $P < 0.01$; femur, $P < 0.01$, $n = 10$). However, raloxifene treatment appeared to significantly increase BMD in both lumbar vertebrae (treated vs. control, 0.077 ± 0.019/0.062 ± 0.011 g/cm^2, $P = 0.02$) and femurs (treated vs. control, 0.084 ± 0.016/0.066 ± 0.012 g/cm^2, $P = 0.02$) of $Opg^{-/-}$ mice albeit significantly lower than that of WT mice (Fig. 2). These results suggest that raloxifene can increase BMD in mice deficient for Opg.

Increased bone trabeculae in $Opg^{-/-}$ mice after raloxifene treatment

The sections of femurs and lumbar vertebrae were stained with Van Gieson's solution, a mixture of acid fuchsin in saturated picric acid solution [16]. Histomorphometrically, mean surface area of bone trabeculae was dramatically decreased in both femurs (Fig. 3a, upper) and lumbar vertebrae (Fig. 3b) of mice deficient for *Opg* as compared with wild-type mice. Obvious bone resorption lacunae caused by osteoclasts can be seen in $Opg^{-/-}$ mice. However, the number and connectivity of bone trabeculae were increased in $Opg^{-/-}$ mice after treatment with raloxifene as compared with control group (Fig. 3a lower, b).

Raloxifene inhibits osteoclasts in *Opg*-deficient mice

Osteoclasts in lumbar vertebrae and femurs were visualized by tartrate-resistant acid phosphatase (TRAP) staining. As shown in Fig. 4a, osteoclasts in $Opg^{-/-}$ femurs are easily discernable, and appear to decrease in number upon raloxifene treatment. The increased osteoclasts may lead to enhanced bone resorption which results in significant loss

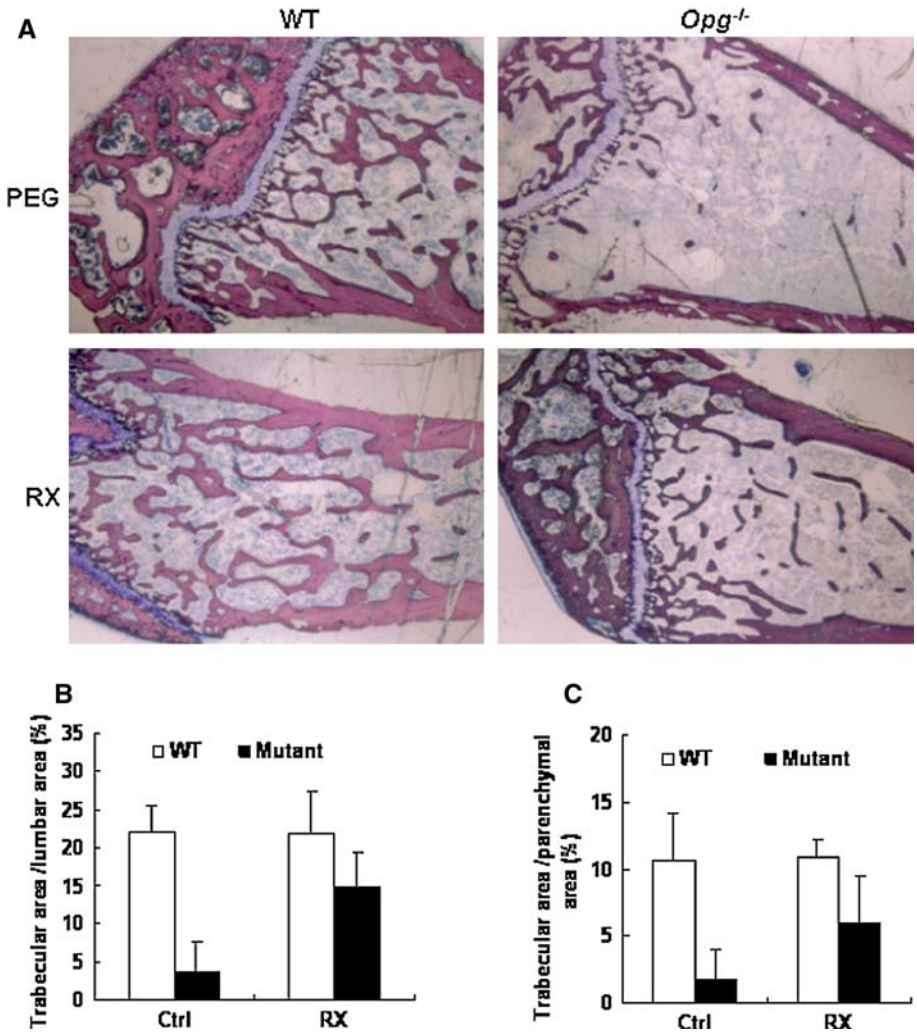

Fig. 3 Raloxifene increases the density of bone trabeculae in $Opg^{-/-}$ mice. **a** Sections of femurs stained with Van Gieson's solution, showing the reduced bone trabeculae in $Opg^{-/-}$ femurs and improvement upon raloxifene treatment. Original magnification 40×. The ratios of bone trabeculae/lumbar vertebrae area (**b**) and bone trabeculae/parenchymal bone area in femurs (**c**) were calculated. The values are the mean ± SD ($n = 10$). $P < 0.01$ ($Opg^{-/-}$ vs. WT), $P < 0.05$ ($Opg^{-/-}$ mice treated with raloxifene vs. controls)

of bone mass. Quantitative analysis revealed that raloxifene induced a decrease in either osteoclast area (Fig. 4b) or number (Fig. 4c) in both lumbar vertebrae and femurs of $Opg^{-/-}$ mice when compared to control. These data suggest that anti-resorptive effect on the bone of raloxifene is not dependent on the presence of Opg.

Raloxifene improves the biomechanical parameters in $Opg^{-/-}$ mice

Increased bone density cannot completely represent the increase of bone mass and decrease of fracture risk. To evaluate the bone quality, bone strength was measured. As shown in Table 1, ultimate load, ultimate stress, and Young's modulus of $Opg^{-/-}$ mice were significantly decreased as compared with those of wild-type mice. After treatment with raloxifene, the values of these indexes were significantly increased for both lumbar vertebrae and femur bones. The results suggest that raloxifene is effective in increasing bone quality and in reducing the risk of fracture even without Opg.

Raloxifene suppresses Rankl expression in *Opg*-deficient mice

The expression of *Opg* and *Rankl* mRNA in femur tissues was evaluated by semi-quantitative RT-PCR and normalized to β-actin. *Opg* was undetectable in $Opg^{-/-}$ mice as expected. However, its expression level in WT mice seemed to decrease after raloxifene treatment. Interestingly, it was found that *Rankl* was significantly increased in $Opg^{-/-}$ mice compared to WT mice ($P < 0.01$), and declined in the raloxifene-treated mice ($P = 0.02$) (Fig. 5a, b). Similar results were obtained by ELISA assay for Opg and Rankl levels in mouse sera. It was found that serum Rankl was significantly higher in $Opg^{-/-}$ mice than that in WT mice ($P < 0.01$), but reduced after treatment with raloxifene ($P < 0.05$) (Table 2).

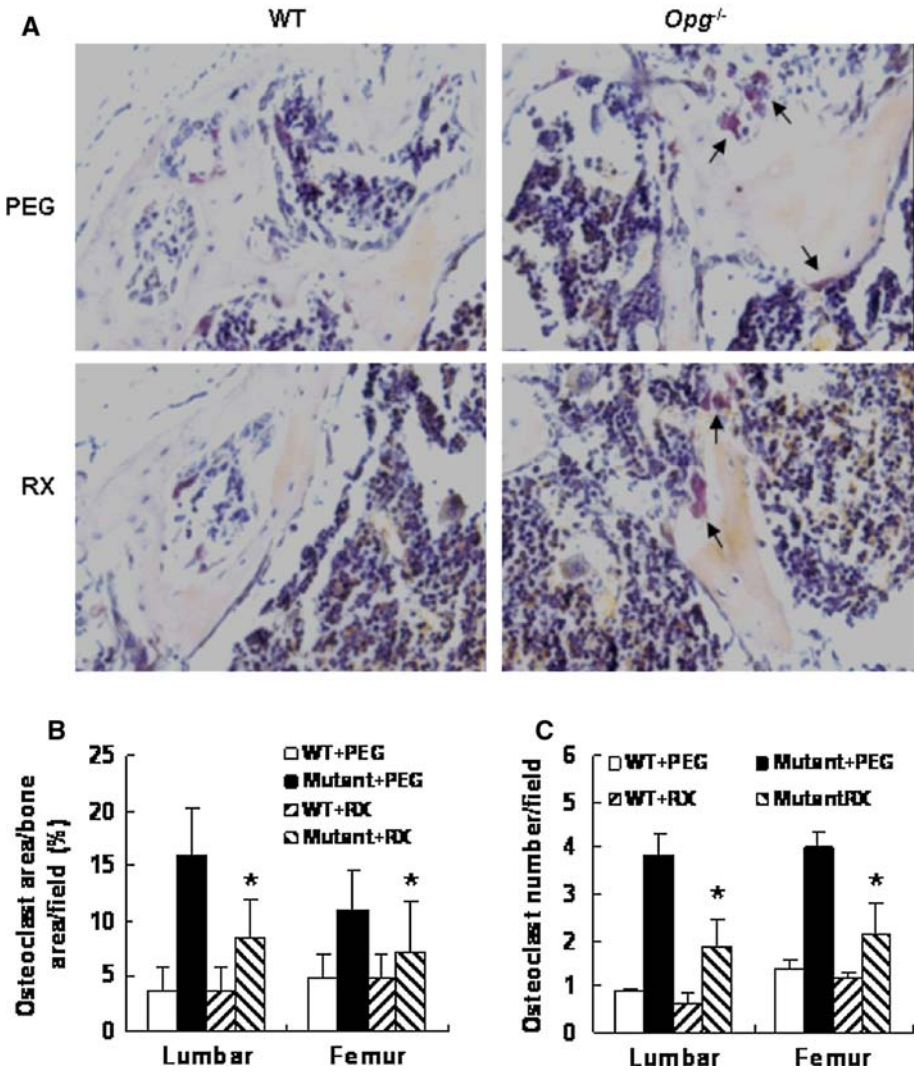

Fig. 4 Decreased osteoclast number in $Opg^{-/-}$ mice upon raloxifene treatment. **a** Femur sections stained with tartrate-resistant acid phosphatase (TRAP) for osteoclasts (*arrows*). Original magnification 200×. Statistical data are shown as the ratios of osteoclast area to bone area per field (**b**) and the number of osteoclasts in each field (**c**). The results are expressed as mean ± SD ($n = 9$). * $P < 0.05$ ($Opg^{-/-}$ mice treated with raloxifene vs. controls)

Table 1 Biomechanical parameter changes in $Opg^{-/-}$ mice upon raloxifene treatment

	Ultimate load (*N*)		Young's modulus (Gpa)		Ultimate tress (Mpa)	
	Lumbar	Femur	Lumbar	Femur	Lumbar	Femur
WT + PEG	66.71 ± 14.44	15.04 ± 1.58	24.20 ± 9.02	12.41 ± 0.57	0.73 ± 0.26	13.88 ± 2.10
WT + RX	72.52 ± 18.38	11.4 ± 4.80	25.98 ± 6.26	12.39 ± 0.33	0.91 ± 0.23	15.96 ± 3.40
$Opg^{-/-}$ + PEG	20.53 ± 5.06$^{\#\#}$	7.28 ± 5.17$^{\#}$	2.2 ± 1.22$^{\#\#}$	3.78 ± 2.37$^{\#\#}$	0.26 ± 0.06$^{\#\#}$	9.88 ± 2.90$^{\#\#}$
$Opg^{-/-}$ + RX	56.29 ± 18.31**	11.68 ± 8.17*	12.01 ± 4.43*	10.1 ± 0.80*	0.7 ± 0.23**	16.00 ± 1.03*

Note that the values are expressed as mean ± SD ($n = 10$)

$^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ ($Opg^{-/-}$ vs. WT)

* $P < 0.05$, ** $P < 0.01$ ($Opg^{-/-}$ mice treated vs. controls)

Discussion

As a decoy receptor, OPG inhibits osteoclast differentiation by binding to RANKL expressed on the surface of osteoblasts, causing reductive bone resorption and augmentative bone mass and bone density. The balance between RANKL-mediated bone resorption and OPG-mediated bone formation is disturbed in the absence of OPG activity. The common result is osteoporosis caused by excessive osteoclast number and relatively low osteogenesis. *Opg* knockout mice provide a model for observing the functions of Opg in vivo. These mice exhibit progressive, intensive

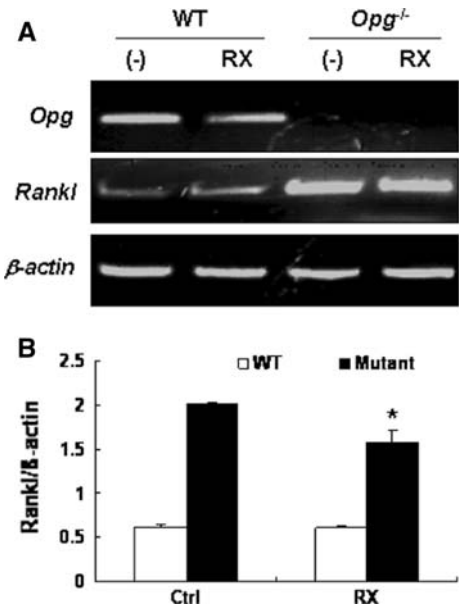

Fig. 5 Raloxifene suppresses *Rankl* expression in $Opg^{-/-}$ femurs. **a** Total RNA extracted from mouse femurs was used to analyze the expression levels of *Opg* and *Rankl* by semi-quantitative RT-PCR by using specific primer pairs. β-Actin is shown as loading control. **b** Band density was normalized with that of β-actin. The data are expressed as mean ± SD ($n = 5$). * $P < 0.05$ ($Opg^{-/-}$ mice treated vs. controls)

Table 2 Serum levels of Opg and Rankl in $Opg^{-/-}$ mice

	Opg (pg/ml)	Rankl (pg/ml)
WT + PEG	1086.80 ± 117.6	115.22 ± 38.7
WT + RX	1359.10 ± 316.2	100.20 ± 40.7
$Opg^{-/-}$ + PEG	0.00	2360.76 ± 269.7**
$Opg^{-/-}$ + RX	0.00	1654.06 ± 117.1*

Note that the values are expressed as mean ± SD ($n = 10$)
** $P < 0.01$ ($Opg^{-/-}$ vs. WT), * $P < 0.05$ ($Opg^{-/-}$ mice treated vs. controls)

high-turnover bone loss, and display characteristics of normal reproduction and growth. Therefore, this animal model is ideal for investigating whether raloxifene regulates bone metabolism depending upon the *Opg* pathway in vivo [17]. In this study, we found the bone loss in $Opg^{-/-}$ mice to be inhibited by raloxifene. The data showed that the manifestations of bone loss were improved in raloxifene-treated $Opg^{-/-}$ group as compared with that in placebo control: bone density was increased in both femurs and lumbar vertebrae; the resulting bone biomechanics measurements including ultimate load, ultimate stress, and Young's modulus (the index of bone strength) were all increased; the number of osteoclasts decreased significantly; histomorphometric measurements showed that the area of bone trabeculae increased. All these findings indicate that the treatment with raloxifene was still effective in $Opg^{-/-}$ mice. It appears that the therapeutic effect of raloxifene on bone loss is independent of *Opg*. Serum Opg and *Opg* mRNA expression in femur tissues were not detectable in $Opg^{-/-}$ mice, accompanied by significantly increased levels of serum Rankl and its mRNA expression in femur tissues. After treatment with raloxifene, Rankl was inhibited in both serum and femur bones, once again, indicating that raloxifene inhibits bone resorption independent of *Opg*.

Estradiol modulates the activity of target cells by binding specific intracellular estrogen receptors (ERs), ERα and ERβ, and trans-activating responsive genes [18–20]. It has been reported that both ERα and ERβ are present in osteoclasts [21–23]. As an estrogen agonist, raloxifene may act similarly to estrogen. It could inhibit osteoclastogenesis and bone resorption directly through ERs without *Opg*. Osteoclasts have been studied extensively as targets for anti-osteoporotic drugs such as SERM. In fact, two other cell types in bone, osteoblasts and osteocytes may be modulated by raloxifene in bone homeostasis. Taranta's study demonstrated that raloxifene stimulates osteoblast activity by increasing the osteoblast-specific transcription factor Cbfα1/Runx2 [10]. These results have been confirmed in human studies. Increased osteocyte apoptosis, as a result of estrogen deficiency, could play a role in the decrease of bone mass and bone strength seen in postmenopausal osteoporosis. Mann et al. [24] investigated the potential antioxidant effects of raloxifene in the prevention of oxidative stress-induced apoptosis in the osteocyte-like cell line MLO-Y4. The results showed that raloxifene significantly inhibits H_2O_2-induced apoptosis in these cells.

Recently, it was found that raloxifene could inhibit tumor necrosis factor-α, interleukin-6, and interleukin-1β production by human trabecular osteoblasts [12, 13, 25]. These cytokines were proven to be associated with osteoclastogenesis. Moreover, raloxifene was found to inhibit the signal pathway downstream of RANK in osteoclastic lineage cells by repressing c-Jun [7]. Our data suggest that other cytokines or factors could play a pivotal role in the mechanisms of the osteoprotective effect of raloxifene. In summary, we have shown that raloxifene can inhibit bone resorption in Opg-deficient mice. The anti-osteoporotic effect of raloxifene is through an Opg-independent mechanism.

Materials and methods

Animals and drugs

Opg-deficient mice and wild-type mice were provided by Shanghai Research Center for Model Organisms [15]. The

animals were housed under specific pathogen-free (SPF) conditions. In this study, $Opg^{-/-}$ and WT mice with a mixed C57BL/6J×129/SV background. Prior to experiments, the genotypes were confirmed by PCR as described [15]. All studies were performed with the approval of the Experimental Animal Committee at our university.

Twenty 3-month-old wild-type or $Opg^{-/-}$ female mice were chosen and randomly divided into raloxifene-treated group and placebo control group. Mice were given raloxifene dissolved in polyethylene glycol (PEG-300, 3 mg/kg/day) or PEG-300 orally for 1 month. All mice were weighed every week to adjust the dose. After these treatments, mice were killed after fasting for 12 h. Sera were collected and stored at −80°C. The left femurs and lumbar vertebrae (L2–L4) were dissected and used to determine the bone density and to perform biomechanical measurements. The right femur slides were prepared and stained with tartrate-resistant acid phosphatase.

Raloxifene was purchased from Sigma (St. Louis, MO). It was dissolved in PEG-300 (Sigma) to prepare the injection solution. These solutions were stored in the dark. Other chemicals and reagents were of analytical grade.

Bone densitometry measurement

The left femur and lumbar vertebrae were fixed onto the scanning table along the longitudinal axis, and were scanned by dual-energy X-ray absorptiometry (DXA) using a PIXImus densitometer (GE Lunar, Madison, WI). The intra- and inter-assay coefficients of variation (CV) were less than 3.6% and less than 2.5%, respectively.

Bone histomorphometry and histological analysis

Ten mice of each group were killed on day 30 for bone histomorphometric analysis. Their femurs and vertebrae were then removed, fixed in 70% ethanol, and embedded in glycol–methacrylate without decalcification. Sections were prepared and stained with fuchsin picric to discriminate between mineralized and unmineralized bone and to identify cellular components. Quantitative histomorphometric analysis was performed in a blinded fashion. Nomenclature and units were used according to the recommendation of the nomenclature committee of the American Society for Bone and Mineral Research [16]. The right femurs were removed and immersed immediately in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) at 4°C. After specimens were washed with phosphate buffer, they were decalcified in 10% EDTA-2Na in 0.1 M Tris buffer (pH 7.3) for 4 weeks at 4°C. Decalcified specimens were then embedded in paraffin and sectioned on a microtome. Tartrate-resistant acid phosphatase, a marker enzyme of osteoclasts, was detected using enzyme histochemistry with naphthol AS-MX phosphate (Sigma–Aldrich Corp.) as a substrate and Fast Violet LB salt (Sigma–Aldrich Corp.) as a dye as previously described [15].

Bone biomechanics analysis

The left femurs were used for the three-point bend testing that was performed using a computer-controlled mechanical testing machine (Instron-5543, USA) equipped with a 500N M-SI sensor (Celtron) under the following conditions: sample space, 9 mm and plunger speed, 1.8 mm/min. The load-deformation curve was plotted, and based on this curve, the bone biomechanical indexes, including the ultimate load, ultimate stress, and Young's modulus, were calculated. Vertebral bone breaking force was measured in lumbar vertebrae (L2) after the vertebra was dissected away and cleaned of soft tissues. The transverse and posterior processes were removed, and the ends of the centrum were made parallel using a diamond wafering saw (Buehler Isomet). The vertebral bodies then were broken in compression along the S–I axis at 37°C using the materials testing machine. The load-deformation curve was plotted, and based on this curve, the bone biomechanical indexes, including the ultimate load, ultimate stress, and Young's modulus, were calculated.

Detection of *Opg* and *Rankl* mRNA expression

Mouse tibias were dissected and total RNA was extracted with Trizol (Invitrogen) according to a standard method. Reverse transcription was performed with 2 μg of total RNA. Semi-quantitative PCR was performed to determine the expression of *Opg* and *Rankl*. Two primers for *Opg*: upstream 5′-TGA GTG TGA GGA AGG GCG TTA-3′, downstream 5′-CCA TCT GGA CAT TTT TTG CAA A-3′. Two primers for *Rankl*: upstream 5′-GCA CAC CTC ACC ATC AAT GCT-3′, downstream 5′-GGT ACC AAG AGG ACA GAG TGA CTT TA-3′.

Measurements of serum Opg and Rankl

Serum Opg concentration was measured by ELISA using a polyclonal anti-mouse Opg antibody as a capture antibody in combination with a biotinylated anti-mouse Opg polyclonal detection antibody (R&D Systems, UK). The inter-assay CV ranged from 3.1 to 5.4% and intra-assay CV ranged from 2.5 to 4.5%. Serum Rankl concentration was also measured by ELISA using the sRANKL kit (R&D Systems, UK). The inter-assay precision ranged from 6.5 to 7.9% and the intra-assay precision ranged from 2.2 to 8.0%.

Statistical analysis

Data are represented as mean ± standard deviation ($n = 10$). Statistical significance between any two groups was determined by a 2-tailed Student t test. P values less than 0.05 were considered to be significant.

Acknowledgment This work was partially supported by the grant of Science and Technology Commission of Shanghai Municipality (06ZR14139, 09GC1410300).

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